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Longitudinal Tracking of Cytokines after Acute Exposure to Tuberculosis: Association of Distinct Cytokine Patterns with Protection and Disease Development[▽]

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Household contacts (HCs) of patients with tuberculosis (TB) are at higher risk of infection as well as the development of active disease. Longitudinal tracking of antigen-specific cytokines after acute exposure may significantly advance our understanding of the dynamic changes in cytokine patterns associated with disease establishment. To achieve this objective, we carried out a prospective cohort study with healthy HCs after exposure to TB. The patterns of cytokines (gamma interferon [IFN- γ] and interleukin 10 [IL-10]) in response to mycobacterial antigens (culture filtrate [CF] proteins) and nonspecific mitogens (phytohemagglutinin [PHA] and lipopolysaccharide [LPS]) were assessed at 0, 6, 12, and 24 months after exposure. Seven of 109 (6.4%) HCs developed active disease. Six of the seven individuals were females, and active disease developed between 12 and 15 months after exposure in 5/20 families. The most significant findings were the exponential increases (~1,000-fold) in both the CF protein- and the PHA- or LPS-induced IFN- γ /IL-10 ratio in healthy HCs ($n = 26$), which peaked at 12 months, compared to the levels in HCs who developed disease ($n = 7$), in whom relatively flat responses were observed during the 24-month period. Linear trends for 0 to 12 and 0 to 24 months for the CF protein-induced IFN- γ /IL-10 ratio showed significant differences between the two groups, as determined by the use of the Mantel extension test for χ^2 analysis (odds ratio = 0.45; 95% confidence interval = 0.295 to 0.685; $P = 0.0002$). Our results strongly suggest that the magnitude of the IFN- γ /IL-10 ratio at 12 months after exposure may be a critical determinant in the resolution of infection. These studies provide new insights into the cytokine responses associated with disease establishment or the resolution of infection after natural exposure to TB and have implications for TB control programs as well vaccine efficacy studies.

Pakistan ranks seventh globally in terms of the tuberculosis (TB) disease burden, with an incidence of 181/100,000 population/year and a prevalence of 329/100,000 population/year (48). Several reports from different countries have shown that the household contacts (HCs) of patients with active pulmonary TB are at a much higher risk of infection, which ranges from 30 to 80%, depending on the intensity of TB disease transmission (1, 2, 5, 16, 24, 33, 40). Most of the newly infected contacts of patients with TB contain the infection and do not develop disease. A small percentage of infected cases, however, go on to develop progressive disease, usually in the first 2 years after exposure (6, 12). The identification of these high-risk individuals among recently exposed or infected individuals is of great importance to TB control programs for reducing the disease burden in the community. Several environmental and host factors have been shown to be associated with susceptibility to TB disease (for a review, see reference 34). Among the host factors, T-cell cytokines and, in particular, gamma interferon (IFN- γ) play key roles in determining susceptibility to TB disease (30, 37), disease severity (15, 27, 39, 45, 51), and the treatment outcome (3, 9, 10, 20, 42). However, the roles of cytokines in established disease do not reflect the dynamic

changes in the immune response in association with disease containment and/or progression to active TB and still need to be determined. An understanding of the ideal repertoire of the immune response in patients with chronic infections such as TB is also of major importance for current vaccine studies.

Longitudinal analyses of cytokines in humans have been limited to studies of individuals before and after *Mycobacterium bovis* BCG vaccination (7) in the context of a point source of exposure in a school TB outbreak in those who recently acquired infection (19) or in the context of latent *Mycobacterium tuberculosis* infection pre- and posttreatment (11, 36, 49). Only one study analyzed cytokine patterns in relation to the incidence of TB disease (17), but that was in the context of human immunodeficiency virus (HIV) infection, which itself could be a confounding factor in the assessment of the immune response. Our objective was to carry out a prospective cohort study of patients with infectious cases of *M. tuberculosis* infection and their contacts after exposure to the disease in Pakistan, which still has a relatively low incidence of HIV infection (48), to identify the cytokine patterns associated with disease development or resolution. The cytokines that we have focused on are (i) IFN- γ , a proinflammatory cytokine important in restricting the replication of *M. tuberculosis* in the macrophages (13, 22) and a critical determinant of susceptibility to mycobacterial infections in humans (30, 37), and (ii) interleukin 10 (IL-10), a down-regulatory cytokine which has been shown to be associated with disease progression (8, 44).

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MATERIALS AND METHODS

Study subjects. HCs ($n = 109$) from 20 families with an index case of infectious pulmonary TB were enrolled in a prospective cohort study between November 2001 and January 2003 and were monitored until January 2005. The patients with TB were >18 years of age and had one or more HCs living with them. Patients were recruited at the Masoomen General Hospital, located in a low-socioeconomic, periurban area of Karachi, Pakistan. The severity of the pulmonary TB disease was based on lung tissue involvement and was classified as minimal, moderate, or advance, as described previously (25). Tuberculin skin test (TST) positivity (TST⁺) was assessed by administering five tuberculin units intracutaneously on the volar surface of the right arm. An induration of ≥ 10 mm after 48 h was considered TST⁺. There was no history of HIV infection in these 20 families. Lady health visitors (LHVs) made home visits during the first week of diagnosis, provided information about TB and the study, and obtained informed consent (written or oral) from all adult participants or the guardians of children less than 18 years of age. Initial examination of the HCs was carried out by one of the authors (G.D.), who documented the health of the participants. LHVs subsequently used a standard questionnaire for clinical evaluation at 3-month intervals. If any contacts showed clinical signs suggestive of TB, additional diagnostic tests, (microscopy and radiology) were carried out to confirm the diagnosis. All active cases were treated with the standard short course of therapy with four drugs (ethambutol, isoniazid, rifampin, and pyrazinamide). Blood samples were collected at intake and subsequently at 6, 12, and 24 months for the assessment of cytokines. The study protocol received the approval of the Ethical Review Committee of The Aga Khan University.

Reagents. *M. tuberculosis* purified protein derivative was obtained from the Statens Serum Institut (batch RT47; Copenhagen, Denmark). Lipopolysaccharide (LPS) and phytohemagglutinin (PHA) were purchased from Sigma Chemical Co. (St. Louis, MO). *M. tuberculosis* culture filtrate (CF) proteins were prepared as described previously (32, 46) and contained minimal amounts of endotoxin (43), as determined by the *Limulus* amoebocyte lysate assay (Sigma). Antibody pairs for cytokine detection were purchased from PharMingen (San Diego, CA).

Stimulation of WB cultures. A stimulated whole-blood (WB) culture assay for assessment of the cytokine profiles has previously been described in detail (26). Briefly, 5 ml blood was collected by venipuncture from each donor, immediately mixed with sodium heparin (20 U/ml; Leo Pharmaceuticals, Ballerup, Denmark) in 50-ml plastic centrifuge tubes, and further diluted 1/11 with sterile RPMI 1640 tissue culture medium containing 100 U/ml of penicillin–100 μ g/ml streptomycin and 2 mM L-glutamine (Sigma). Diluted WB (900 μ l/well) was dispensed in 24-well tissue culture plates (Flow Laboratories, Irvine, CA) within 2 h of collection. The cultures were stimulated with 100 μ l/well of mitogen (final concentrations, PHA, 5.0 μ g/ml; LPS, 1.0 μ g/ml) or antigens of *M. tuberculosis* CF proteins (final concentration, 5 μ g/ml) for either 2 or 5 days. The plates were incubated at 37°C in 5% CO₂. Supernatants were collected from the wells and stored as four 200- μ l aliquots at -35°C .

Cytokine determinations in stimulated WB cultures. The levels of the cytokines (IFN- γ and IL-10) in the supernatants were assessed with pairs of monoclonal antibodies by enzyme-linked immunosorbent assay (ELISA)-based assays. Probing antibodies labeled with biotin and revealing antibody labeled with avidin were used in the assay. The assay was optimized in-house, and both the sensitivities and the ranges of the assays were determined by running a full dose-response curve of a reference standard from the manufacturer on each plate. The sensitivity was 7.5 pg/ml, and the range of cytokine detection was 7.5 to 1,000 pg/ml, which were comparable to those reported by the manufacturer. Cytokine results are expressed as the change in pg/ml (δ pg/ml), after the spontaneous secretion in the absence of a stimulant was deducted. The CF protein-induced IFN- γ /IL-10 ratio was derived by dividing the IFN- γ (pg/ml) value by the IL-10 (pg/ml) value for each donor before further analyses were carried out.

Statistical analysis. SPSS software (version 13.0) was used for the statistical analyses. The Fisher exact test or the Mann-Whitney U test was applied, as appropriate, to compare the significance of the difference between different groups for demographic, TST⁺, and cytokine responses. The EPI-Info software program (version 6.0) was used to determine χ^2 (Mantel extension) for trends to calculate the odds for an increase or a decrease in the geometric means in successive groups. A P value of <0.05 was considered a reasonable indication of a trend in the odds of successive levels compared to those at the baseline (0 months). GenoPro 2007 software was used to generate family pedigrees.

TABLE 1. Dynamics of disease development and longitudinal cytokine assessment timeline

Time (mo) of follow-up ^a	No. of new cases postexposure	Clinical follow-up	Follow-up for cytokine analysis
0 (109)	0	✓	✓
3 (84)	0	✓	
6 (92)	1	✓	✓
9 (67)	0	✓	
12 (94)	3	✓	✓
15 (66)	2	✓	
18 (53)	0	✓	
21 (78)	0	✓	
24 (93)	0	✓	✓
33 (1)	1	✓	✓
Total	7		

^a The values in parentheses are the numbers of donors from whom clinical and/or cytokine information was available.

RESULTS

Dynamics of disease development in HCs. A cohort of 109 HCs from 20 families containing one index case per family with sputum-positive open pulmonary TB disease were prospectively monitored for 2 years. The index cases had a history of 1 to 4 months of cough, chest pain, fever, and weight loss and had received <25 days of antituberculous treatment at the start of the study. The HCs were monitored at regular intervals for 24 months for the development of signs and symptoms of clinical disease and cytokine assessment (Table 1). Two families ($n = 17$ individuals) were lost to follow-up at 3 months due to migration. Ten HCs had received prior antituberculous treatment, and these 10 HCs, 9 remained disease-free during the monitoring period. Seven HCs (one of whom had been treated previously) from 5/20 families developed active disease during the follow-up. The incidence of new cases was therefore 6.4%. Although the sample size is small, this incidence is consistent with the incidences, reported in a much larger studies of HCs, of 4.5% in India (16), 3.7% in Brazil (33), and 6.0% in Uganda (24). The majority (five of seven) of the contacts developed disease from 12 to 15 months postexposure, as has been reported previously in a much larger study (12).

Description of families with new cases of TB. Figure 1a to c show the pedigrees of each of the five families with new incident cases. The segregation of TB status for each individual is shown by different symbols and included (i) the index case, (ii) new incident cases, (iii) previously treated individuals who remained disease-free during the 24 month follow-up, and (iv) disease-free individuals who never had TB. Three of the seven new cases belonged to a single family (Fig. 1c, family 19), with only the female members of the family developing disease. One of the newly diagnosed cases had received a full-course TB regimen 6 months earlier (Fig. 1b, family 16, individual HC146) and developed active disease again at 12 months. Similarly, there were two individuals (individuals HC130 and HC149) who had previously been treated for TB but who remained disease-free throughout the 24-month follow-up period, although one new case was detected in each of these families (individuals HC132 and HC146). In family 5 (one of the families that enrolled early in the study), there was one new case who self-reported to the clinic at 33 months. TB disease

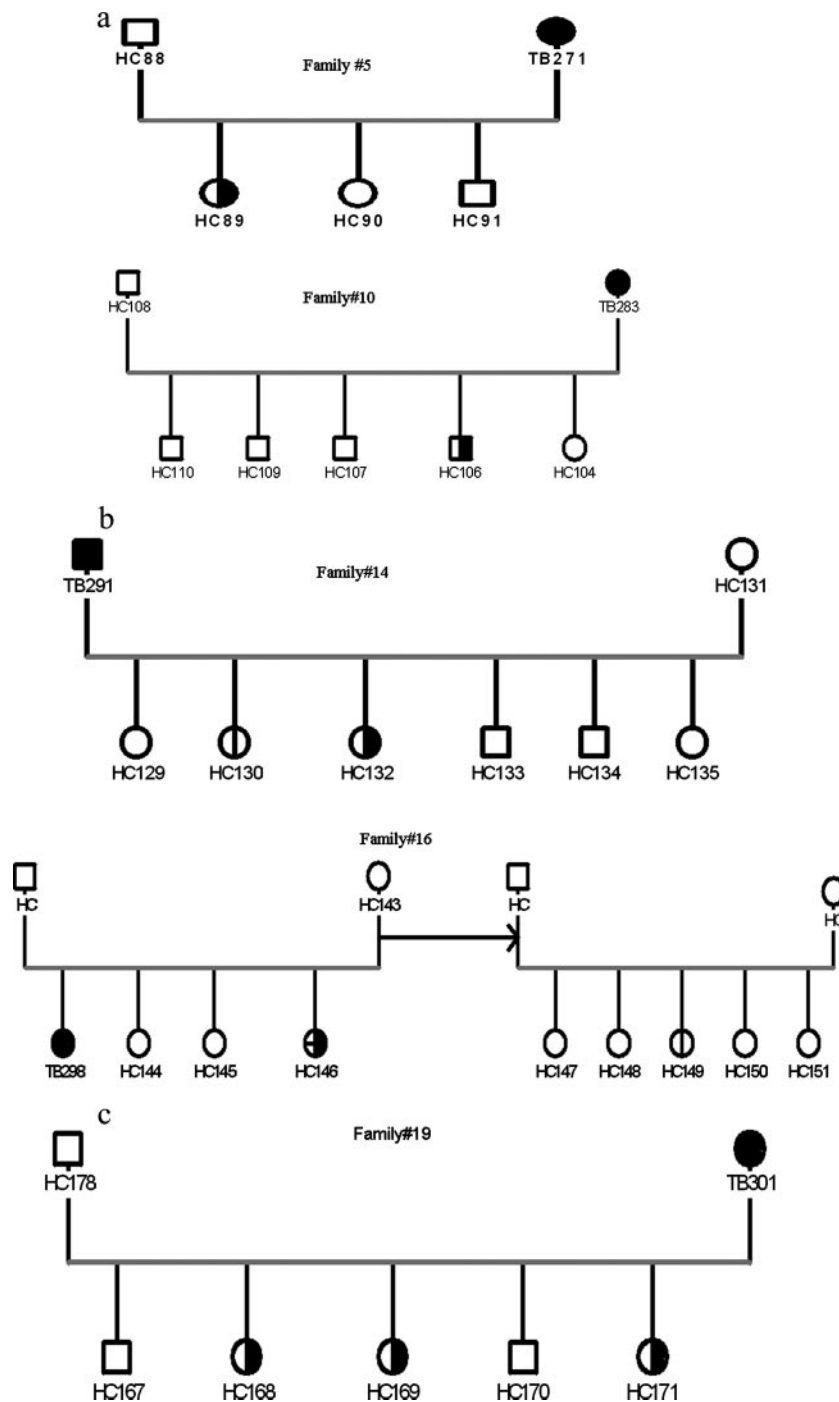


FIG. 1. Pedigrees of five families with new active TB cases. Squares, males; circles, females; filled symbols, cases; half-filled circles and squares, newly detected TB cases; horizontal line in unfilled symbols, previously treated patient; vertical line in half-filled circles, previously treated and reactivated TB cases. The pedigrees were generated with GenoPro 2007 software.

susceptibility was significantly associated with female gender in these five families ($P = 0.045$; Fisher exact test) (Table 3) and is consistent with the findings presented in previous reports of studies performed in this setting (25) and recently from Lima, Peru (5). The case notification for 2004 in Pakistan (48) also shows a trend for a slightly higher incidence of TB among females in the first three decades of age. The reasons for this

gender-related difference in susceptibility to TB are unclear. One likely reason in this particular community may be the lifestyle, in which male members are fishermen by profession and usually leave home before sunrise and return late in the evening. Female members within the 15- to 25-year-old age group (a group at high risk for TB) stay at home. Since three of the five index cases in these families were mothers, it is likely

TABLE 2. Clinical characteristics of newly diagnosed tuberculosis patients

Diagnostic parameter	Clinical characteristic	No. of patients positive/no. of patients tested
Symptoms	Cough	7/7
	Chest pain	7/7
	Fever	7/7
	Night sweats	5/7
	Weight loss	6/7
Physical signs	Rales	1/7
	Dullness	2/7
	Pleural effusions	2/7
Radiological lesions	Calcified	6/7
	Localized	2/7
	Diffused	4/7
	Hilar lymph nodes	3/7
	Pleural effusions	2/7
Laboratory tests	Sputum <i>M. tuberculosis</i> positive	1/7
	Raised ESR ^a	7/7
Disease classification	PMN ^b	4/7
	PMD ^c	3/7

^a ESR, erythrocyte sedimentation rate.^b PMN, minimal lung disease.^c PMD, moderate lung disease.

that the females had more intense exposure, which may be a contributor to the uneven gender distribution, although genetic factors cannot be excluded at this stage.

Clinical characteristics of newly diagnosed TB patients.

Clinical signs, raised erythrocyte sedimentation rates, and radiological lesions in the lung were present in all patients (Table 2). Only one patient (patient HC089) was sputum positive for acid-fast bacilli. This patient had moderately advanced pulmonary disease and had self-reported 33 months after exposure, indicating that there may have been a delay in diagnosis since the last follow-up of this family had been done at 24 months, at which time this family had been released from follow-up. Interestingly, four of the seven patients had minimal pulmonary disease and none had advanced lung disease, findings which are consistent with the early case detection after a point-source exposure. Several patients (three of seven) showed hilar lymph node involvement, and in addition, two patients showed pleural effusions. All seven cases showed radiological and clinical improvement within 6 months of chemotherapy. However, three female members belonging to the same family (family 19) showed a recurrence of symptoms at the 24-month follow-

up. It is difficult to evaluate whether these were reactivation cases or exogenous reinfections due to a highly susceptible genetic background. None of the newly diagnosed patients in family 19 (ages 12 to 15 years) had received prior TB treatment, and all three patients became asymptomatic within 6 months posttreatment. It is therefore unlikely that these patients had drug-resistant TB or that the recurrent TB was due to noncompliance with a previous treatment regimen. We are analyzing cytokine gene polymorphisms associated with the susceptibility to TB in these families to understand the reasons for such a susceptible background.

In order to restrict the socioeconomic, genetic, and environment variabilities further, we compared the immunological responses within the members of these five families. Table 3 compares the ages, genders, and TST⁺ status of the new incident cases (HCs with TB disease [DHCs]; $n = 7$), the disease-free HCs (healthy HCs [HHCs]; $n = 26$), and the index cases pretreatment (TB cases; $n = 5$). The rates of TST⁺ (>80%) were comparable in all three groups, reflecting the high rate of transmission in these families. A slightly lower mean diameter of the indurations was observed in HHCs, but there were no significant differences among the groups ($P = 0.084$; Fisher exact test). These results indicate that TST⁺ or BCG vaccination in exposed HCs in a region with high rates of TB transmission do not influence the outcome of disease in acutely exposed HCs, as has been shown in a much larger study with an Indian population (16). The greatest risk factor in these families was female gender.

Cytokine secretion profiles in WB of DHCs and HHCs. (i) Kinetics of IFN- γ and IL-10 secretion to different stimuli. To determine the best time points for the comparison of cytokine secretion among the groups, we first assessed the kinetics of cytokine secretion on days 1, 2, and 5 in four HCs at the baseline. Figure 2 shows the kinetics of IFN- γ and IL-10 secretion after stimulation with either mitogen (PHA or LPS) or CF protein. All values are expressed after deduction of the background secretion in the absence of an exogenous stimulus. The kinetics of IFN- γ secretion in response to both stimuli were similar, peaking at day 5 (Fig. 2a), although the magnitude of the response was 10-fold higher for PHA. In the case of IL-10 (Fig. 2b), both the magnitude and the kinetics of IL-10 secretion in response to LPS (monocyte activator) and CF protein were similar (LPS mean = $2,917 \pm 629$ pg/ml; CF protein mean = $1,927 \pm 338$ pg/ml), indicating the potent IL-10-inducing activity of the CF protein in WB cultures, as shown previously (18, 26). This CF protein preparation had minimal detectable LPS (43), and therefore, monocyte activation could not be attributed to endotoxin contamination. PHA was a less potent stimulator of IL-10 than LPS or CF protein,

TABLE 3. Demographic characteristics and TST status in five families with new incident cases

Group	No. of individuals	Age (mean \pm 1 SD) ^a	No. of M/no. of F ^b	% TST ⁺	TST ⁺ induration (mm [mean \pm 1 SD])
Index case	5	34 \pm 5	1/4	80	15 \pm 4
DHC	7	16 \pm 3.46	1/6	85	18.42 \pm 9.27
HHC	23	20.2 \pm 12.16	12/11	83	14.30 \pm 8.16

^a Values are numbers of years.^b M, male; F, female. $P = 0.045$ for the difference in gender, determined by χ^2 analyses by the Fisher exact test for diseased individuals and healthy contacts in the family.

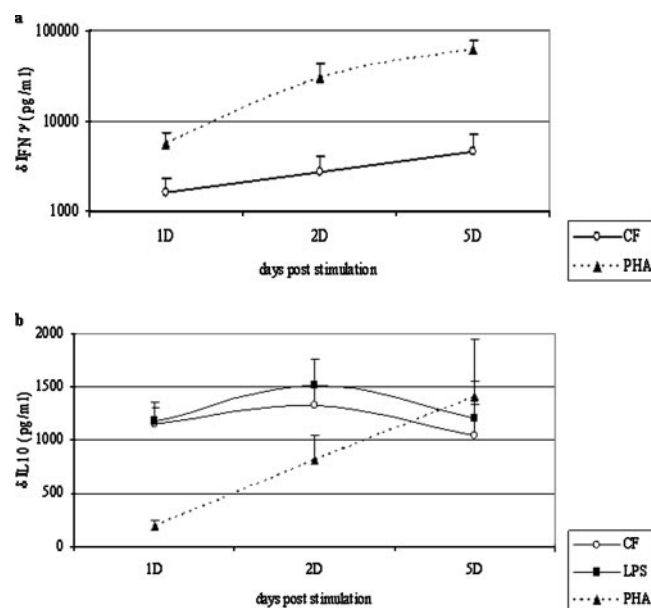


FIG. 2. Kinetics of IFN- γ and IL-10 secretion in stimulated WB cultures. Cultures of WB from healthy HC donors ($n = 4$) diluted 1:11 with RPMI 1640 medium were stimulated with mitogens (PHA [5 μ g] or LPS [1 μ g]) or *M. tuberculosis* CF protein (5 μ g/ml). Supernatants were collected at day 1 (1D), day 2 (2D), or day 5 (5D) for the assessment of IFN- γ (a) and IL-10 (b) by ELISAs. The results are given as the mean pg/ml after deduction of the values obtained in the absence of stimulation. Vertical bars indicate ± 1 standard error of the mean.

and with PHA stimulation IL-10 showed a kinetic pattern (Fig. 2b) that was different from that obtained with LPS stimulation and that was comparable to the kinetics of IFN- γ . The differences in the kinetics of IL-10 secretion to different stimuli suggest different cellular sources for IL-10. IL-10 is secreted by both alternatively activated monocytes (M2 subset) (14, 31, 35) and iTregs (Tr1 subset) (29). Our results suggest that M2 macrophages may be a major cellular source of IL-10 in CF protein-stimulated WB, as it parallels the LPS-induced kinetic pattern rather than the PHA (T-cell mitogen)-induced kinetic pattern. However, this issue needs confirmation with purified cells. In the current study, for analysis of the relationship of cytokine secretion between the different groups, we therefore selected times of peak secretion of day 5 for IFN- γ and day 2 for IL-10.

(ii) Dynamics of CF protein-induced IFN- γ and IL-10 secretion. We next assessed the dynamics of CF protein-induced IFN- γ and IL-10 to see if the evolution of the cytokine responses postexposure had a relationship with disease development (Fig. 3a and b). During the first 6 months after exposure, both DHCs and HHCs showed consistent increases in IFN- γ (expansion phase), with slightly higher responses detected in DHCs. Thereafter, DHCs showed a sharp decline in IFN- γ levels at 12 months postexposure, while the expansion phase continued in HHCs for 12 months postexposure and then dropped to the baseline level at 24 months. CF protein-induced IL-10 levels were consistently higher at all time points in DHCs than in HHCs. However, statistically significant differences (as determined by the Mann-Whitney U test) were ob-

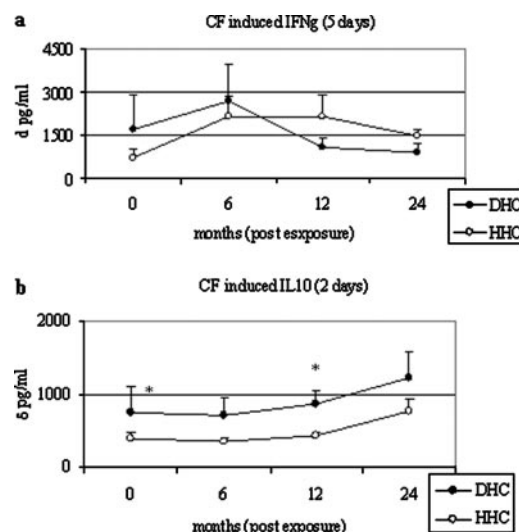


FIG. 3. Dynamic changes in IFN- γ and IL-10 cytokine responses in the newly diagnosed DHC group and the HHC group. The levels of IFN- γ (5 days) (a) and IL-10 (2 days) (b) secretion in CF protein-stimulated cultures of WB from DHCs and HHCs were compared. Data are for 7 DHCs and 26 HHCs at all time points except 6 months, for which the data are for 23 HHCs. The results are given as mean concentrations (pg/ml) after deduction of the values obtained in the absence of stimulation in each group. Vertical bars indicate ± 1 standard error of the mean. All other parameters are same as described in the legend to Fig. 2. *, significant differences ($P = 0.031$ for IL-10 at 0 and 12 months; Mann-Whitney U test).

served only for the IL-10 responses at 0 months and 12 months ($P = 0.03$). The dynamics of the IFN- γ and IL-10 responses and the relationship between IFN- γ and IL-10 were therefore different in the DHC and HHC groups. Since the majority of exposed contacts developed disease from 12 to 15 months postexposure, the sharp decline in IFN- γ levels may be related to either the migration of activated cells to the disease site (lungs) in DHCs or the suppression of T-effector memory (Tem) cells by the expansion of Tregs (4, 41). We have recently shown that the IFN- γ /IL-10 ratio is a critical determinant of disease severity across the TB disease spectrum (27). We therefore analyzed the IFN- γ /IL-10 ratio to see if this ratio is related to disease progression as well.

(iii) Dynamics of antigen- and mitogen-induced IFN- γ /IL-10 ratio. Figure 4 shows the patterns of the IFN- γ /IL-10 ratios in response to mycobacterial antigens (Fig. 4a and b) and mitogens (Fig. 4c and d) in the DHC and HHC groups and their index cases in the five families in which new incident cases were detected. The results are expressed as either pg/ml (Fig. 4a and c) or the percent change (Fig. 4b and d). Surprisingly, the patterns of the responses to both antigen-specific and antigen-nonspecific mitogens were similar. HHCs showed exponential increases in the IFN- γ /IL-10 ratio (1,000% change), which peaked at 12 months and then reached the baseline levels, while the TB index cases and DHCs showed a more or less flat response, with a small rise at 6 months postexposure. Although the magnitudes of the mitogen-induced responses were much higher than mycobacterial antigen-induced responses in terms of the concentrations (pg/ml), the percent

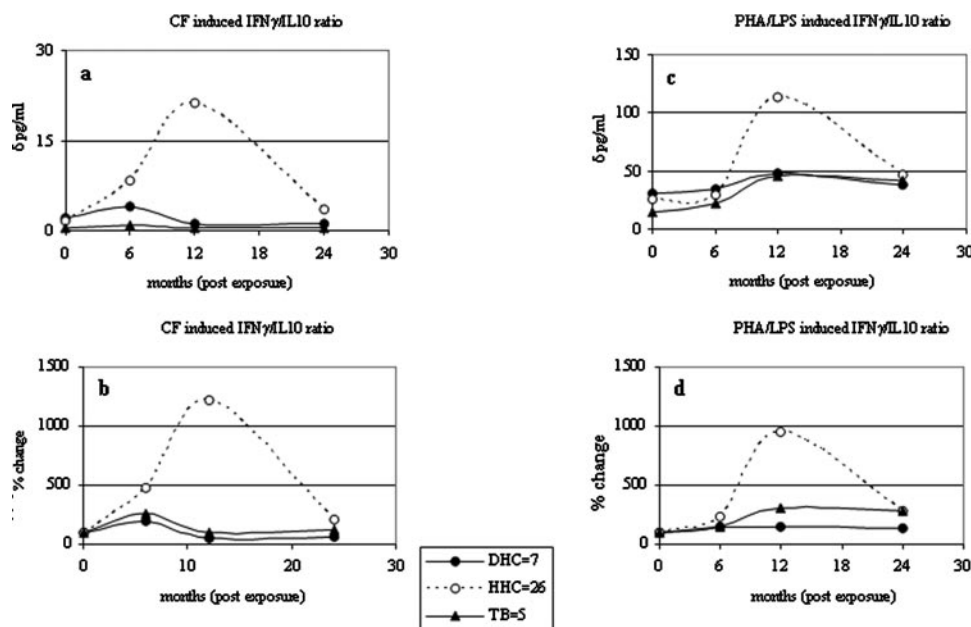


FIG. 4. Dynamic changes in the IFN- γ /IL-10 ratios for DHCs, HHCs, and their index TB cases over a 2-year follow-up. The dynamic changes in the IFN- γ /IL-10 ratio in response to CF protein (a) and PHA and LPS (c) are shown. The numbers in the DHC and HHC groups are the same as those described in the legend to Fig. 3. The data are also for five TB index cases. The IFN- γ /IL-10 ratio was derived by dividing the concentration (pg/ml) of IFN- γ (at 5 days poststimulation) by that of IL-10 (at 2 days poststimulation) in WB cultures for each donor and then calculating the mean level in each group. The percent change in the IFN- γ /IL-10 ratio was calculated relative to the baseline levels, which were considered to be 100%. (b) CF protein induction; (d) PHA and LPS induction. All other parameters are the same as those described in the legend to Fig. 2.

changes relative to the level at the baseline (0 months) showed parallel profiles.

Analysis of the trends of the different cytokine responses in DHCs and HHCs. Statistics were insufficiently powered to be applied to the DHC and HHC groups due to the small sizes of the groups and the variability of the responses among the donors. We therefore compared the trends of the cytokine responses in DHCs and HHCs (Table 4). The trend patterns were compared by applying the Mantel extension test for the χ^2 test to the geometric means of the two groups. The trends for the CF protein-induced IFN- γ /IL-10 ratios were highly significantly different for both 0 to 12 months ($P = 0.0173$) and 0 to 24 months ($P = 0.0002$), while the trend for the mitogen-induced IFN- γ /IL-10 ratio (Table 4) was significant only for 0 to 12 months ($P < 0.0001$). Since mitogens provide a much stronger signal, it is possible that the Tem cells go through an

accelerated expansion phase. Our results provide the first evidence of differences in cytokine profiles in association with the resolution of infection or the progression to disease after exposure to *M. tuberculosis*.

DISCUSSION

The identification of close contacts with latent *M. tuberculosis* infection who are at high risk of disease development is an important priority of TB control programs in countries with a high burden of TB. This is the first report to show highly significant differences in the response patterns in HCs after a point-source exposure to *M. tuberculosis* in association with the resolution or the development of active disease in a BCG-vaccinated, low-HIV-incidence setting (48). In our study cohort the rate of TST⁺ was >80%, but only 6.4% (7/109) of the

TABLE 4. Trend analysis for IFN- γ /IL-10 ratio in DHCs and HHCs

Time postexposure (mo)	CF protein stimulation ^a				PHA stimulation ^b			
	Geometric mean IFN- γ /IL-10 ratio		OR ^d	CI ^e	Geometric mean IFN- γ /IL-10 ratio		OR	CI
	DHCs (n = 7)	HHCs ^c (n = 26)			DHCs (n = 7)	HHCs ^c (n = 26)		
0	0.89	0.75	1		11.95	17.35	1	
6	2.39	3.03	0.67	0.470–0.947	11.15	17.84	0.91	0.655–1.271
12	1.18	1.65	0.60	0.409–0.888	15.06	38.17	0.57	0.425–0.773
24	0.71	1.33	0.45	0.295–0.685	18.18	20.08	1.31	0.966–7.850

^a Statistically significant differences in trend between DHCs and HHCs at 0 to 12 months and 0 to 24 months ($P = 0.017$ and $P = 0.0002$, respectively; χ^2 test).

^b Statistically significant differences in trend ($P = 0.0001$) between DHCs and HHCs at 0 to 12 months.

^c Values were measured for 23 HHCs at 6 months.

^d OR, odds ratio.

^e CI, 95% confidence interval.

HCs went on to develop disease, which indicates the unreliability of TST⁺ as a risk factor for TB disease development in high-transmission regions of endemicity.

A cohort study design such as ours has several advantages over case-control, cross-sectional, and clinical study designs, which involve TB patients with established disease or complications like diabetes or prolonged steroid treatment. The other important aspect of this study is that the community studied still has a low prevalence of HIV infection (48). Our study group had no comorbidities associated with a high risk of disease development, such as diabetes or steroid treatment. However, one new case had uncomplicated malaria at the time of TB diagnosis and was treated with a quinine derivative. We are not sure if this could have been a trigger for the precipitation of TB. Our study was set in a high-transmission area, and therefore, it is likely that the HCs were exposed to TB within the community. In addition, the index cases had various symptoms of TB (for 1 to 4 months). Both of these factors could result in some degree of variability in the immune responses, and such variability is a limitation of clinical studies. However, since the index cases were all *M. tuberculosis* positive at the start of the study, it is very likely that acute and repeated exposures were occurring. In addition, it is unlikely that any of the index cases had drug-resistant TB, as all of them responded to treatment and were microscopically negative after 6 months of treatment.

The initial immune response after exposure may be crucial in determining the clinical outcome of disease. The risk of progression to disease after exposure is the highest during the first 1 to 2 years after exposure (6, 12). We therefore monitored our cohort of exposed HC for 2 years after a known exposure to an index case with open pulmonary disease. In our cohort as well, the peak incidence of new cases occurred between 12 and 15 months postexposure. A clinical evaluation was performed every 3 months. The maximum delay between the time of onset of symptoms and the time of diagnosis was of on the order of 1 to 2 months. Interestingly, one subject had symptoms (fever and cough) 9 months before establishment of the disease. These symptoms disappeared and then reappeared after 6 months (data not shown). This indicates that the progression to disease may be a dynamic process, with a ying-yang effect between resolution and progression in the early stages of the disease, depending on the dynamics of the regulatory mechanisms activated at the initial stage of the disease. This study was set in an area where the population is vaccinated with BCG, and some degree of protective response is expected and may be the reason why a majority of the patients had either minimal (four of seven patients) or moderate disease. BCG vaccination has been shown to have variable protective efficacy for pulmonary disease in different populations (21). Early diagnosis may be another factor for less severe disease. Although TST⁺ is associated with latent infection (28) and is a contributing factor in disease progression, we saw no impact of TST⁺ on disease development in this group, as the TST⁺ status of both HHCs and DHCs was similar (>80%). This may be because TST⁺ represents stable latent infections in populations among whom TB is endemic, while in our cohort we may be looking at the effect of exogenous exposure in TST⁺ HCs. A recent study that used DNA fingerprinting analysis showed a high incidence of exogenous infections in latently infected or

treated patients in a setting where TB is highly endemic (47). The lack of association of TST⁺ and the development of pulmonary disease is well established. However, in our study it is difficult to confirm that exogenous infection was responsible for disease development in the absence of fingerprinting, although the pattern of disease is in line with recent exposure.

For the antigen-induced cytokine responses, we restricted the analysis to these five families to control for variability in both genetic and environmental factors. The IFN- γ /IL-10 ratio was the parameter that showed the most convincing differences between the two groups, with an initial exponential increase in the first 6 to 12 months in HHCs declining to the baseline levels. DHCs showed a flat response throughout the follow-up period. That this particular pattern could be related to disease development is further supported by the similarity of the pattern for the DHCs to that for the TB index cases. The exhaustion of Tem cells has been reported in patients with TB (23). Our results suggest that a similar exhaustion of Tem cells may have occurred in DHCs due to repeated exposure or an overwhelming exposure, resulting in an inability of these individuals to control the infection. DHCs also consistently showed higher levels of IL-10 compared to those detected in HHCs at all time points. IL-10 limits collateral tissue damage, particularly in the lung (38). All of our new cases had pulmonary disease. IL-10 is secreted by both alternatively activated M2 (14) and Tr1 (29) macrophages. M2 macrophages down-regulate classically activated macrophages (31), which are important in limiting mycobacterial replication, while Tr1 down-regulates Th1 (IFN- γ secreting) responses to mycobacterial antigens (4). Both cell types may be important in dampening the proinflammatory arm of the immune response to reduce collateral tissue damage (38). Interestingly, we also observed an increase in the spontaneous secretion of IL-10 at the time of diagnosis of new incident cases (data not shown). This increase may reflect an overall endogenous activation of either M2 cells or Tr1 cells. While the local concentrations of IFN- γ may play a critical role in limiting the growth of mycobacteria at the disease site, the magnitude of the IL-10 response may tip the balance, as reflected by the IFN- γ /IL-10 ratio, toward disease progression by down-regulating both classically activated macrophages and Th1 cells. Of the 20 families inducted into the study, new incident cases developed in 5 families, with one multicase family with three members who developed TB, despite the provision of full anti-TB chemotherapy regimens. These three members showed a highly dysregulated IL-10 response. Further analysis of cytokine gene polymorphisms may shed insights into the genetic markers linked to TB susceptibility in this family.

Although WB assays are simple to perform and are applicable for use in the field, allowing comparative studies to be performed with large cohorts, there are several limitations associated with this assay. One such limitation is the undefined cell source of cytokines. However, this could also be the strength of the assay, as it reproduces the *in vivo* situation more faithfully, since a variety of cells may contribute to a single cytokine (such as IL-10) in the natural host environment. An additional limitation of assessments of cytokines in blood compartments is that they may not truly reflect the activation of different cell types at the disease sites. Cytokine responses at the disease sites have been reported to be two- to

fourfold higher (41, 50), which may result in quantitative differences in cytokines, but the qualitative differences were shown to be unaffected (41). Despite these limitations, we have shown discriminating patterns of cytokine profiles in the blood compartment associated with the resolution of infection or the progression to disease after exposure to *M. tuberculosis* in a natural setting. These studies provide new insights into the immunological correlates associated with disease establishment or the resolution of infection in patients with TB and have implications for TB control programs as well as vaccine efficacy studies.

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